Thrombogenesis of different cell types seeded on vascular grafts and studied under blood-flow conditions

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Background: **Small-diameter vascular grafts tend to have an early and high occlusion rate. Cell seeding on the luminal surfaces of small-diameter vascular prostheses may provide an antithrombotic lining and improve both the short-term and the long-term patency rates. We studied the net results of procoagulant and anticoagulant properties of seeded grafts under blood-flow conditions, and we compared the different available types of donor cells.**

Methods: **Monolayers of liposuction-derived cultured human microvascular endothelial cells (MVECs), human adult endothelial cells (HAECs), human umbilical vein endothelial cells (HUVECs), and human mesothelial cells (MCs) that had been seeded on expanded polytetrafluoroethylene (ePTFE) grafts were perfused with marginally anticoagulated blood (20 U/mL low molecular weight heparin; shear rate, 400/s, 10 minutes) or with non-anticoagulated blood (shear rate, 100/s, 5 minutes). The thrombin and fibrin generation in time was studied with the measurement of the plasma levels of prothrombin fragment 1 and 2 (F 1+2) and of fibrinopeptide A (FPA). The plain ePTFE graft was taken as a control.**

Results: **When the seeded MCs were perfused with recirculating anticoagulated blood, a linear generation of F 1+2 in time was seen, with high levels of F 1+2 and FPA after 10 minutes (4.38 nmol/L and 362 ng/mL, respectively). Allopurinol was added, and the MCs generated less F 1+2 than the HAECs (0.7 nmol/L vs 1.86 nmol/L; $P < .05$). No fibrin formation was seen. The MVECs generated low amounts of F 1+2 (0.7 nmol/L; 10 minutes), and the HUVECs and the plain ePTFE graft generated the lowest amounts of F 1+2 (0.26 and 0.25 nmol/L, respectively). When the MCs were perfused with non-anticoagulated blood, high amounts of thrombin and fibrin were generated immediately and constantly and could not be decreased with allopurinol. The perfusion of the plain ePTFE graft showed a dramatic increase in F 1+2 and FPA levels towards the end of the experiments. The seeded HAECs, HUVECs, and MVECs inhibited this increase. These results were confirmed by means of scanning electron microscopy.**

Conclusion: **Vascular prostheses that are seeded with cultured MCs are highly procoagulant. Standard ePTFE graft prostheses also initiate coagulation, which supports the idea of cell seeding. The endothelial cells, of which the MVECs are the most readily available, seem to preserve their anticoagulant properties after being seeded on vascular grafts.**

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One major problem of small-diameter vascular grafts is the high occlusion rate.[1] Undoubtedly, the best arterial substitute remains the autologous vessel, but only a limited number of suitable arteries and veins are available. Thus, the use of prosthetic small-diameter prostheses with known inferior patency rates is unavoidable.[2,3] Prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade on the graft.[4,5] In humans, re-endothelialization is limited to the ingrowth of approximately 1 cm at each anastomosis.[6] The seeding

of the luminal surface with endothelial cells (ECs) may give a prosthetic graft a biologic lining that might contribute to the balance of the hemostatic process. Unfortunately, adult human HLA-compatible ECs, which are the natural lining of the arteries, are only scarcely available. Most laboratory and clinical experience has been gained with HLA-compatible adult endothelial cells (HAECs) that were harvested from large veins. Cell seeding with HAEC, however, has a major drawback because of the fact that the cell numbers in the primary isolates are not high enough to seed a graft with a confluent layer of cells. So, it becomes necessary to first grow these cells in vitro before they can be seeded on prosthetic grafts. Therefore, attention has turned to alternative cell sources. The mesothelial cells (MCs) are isolated easily in high numbers from the omentum of the candidate for graft implantation, and they grow considerably faster to a confluent monolayer than do ECs. The MCs have hardly any growth failures, and they possess antithrombotic and fibrinolytic properties.[7-12] Recent work in our laboratory showed that the MCs express substantial amounts of tissue factor after isolation and culture, which is in contrast to the in vivo situation.[13,14] With the change of the culture conditions and with the inhibition of the formation of free radicals, their tissue factor expression was inhibited by 90%.[14] Microvascular endothelial cells (MVECs) are abundantly available from subcutaneous fat tissue. The procurement is minimally invasive (liposuction), and the isolated cell numbers are so high that single stage seeding and sodding procedures are possible.[15-20]

From previous studies, we have learned that MCs and MVECs do form a monolayer on fibronectin-coated vascular grafts. This monolayer was shown to be resistant to physiologic shear stress.[21,22] The aim of this study was the examination of thrombin and fibrin formation with MC-seeded and EC-seeded grafts in whole blood under flow conditions.

METHODS

Isolation and culture of cells

Mesothelial cells. Small pieces of human omentum (±20 cm^2) were obtained from patients who underwent abdominal surgery. The pieces of omentum were washed with a phosphate-buffered saline solution (PBS) and were transferred to a prewarmed PBS that contained 0.05% trypsin and 0.02% ethylenediamine tetraacetic acid (Gibco Europe Ltd, Paisley, United Kingdom). After 15 minutes of incubation with gentle shaking at 37°C, the detached MCs were pelleted with centrifugation at 1200 rpm for 8 minutes. The pelleted cells were resuspended in medium 199 (M199), which contained 10% fetal calf serum (FCS; Gibco), and were cultured in 75-cm^2 polystyrene flasks (Costar, Cambridge, Mass), which had been precoated with fibronectin. The cells were grown in a humidified 5%-CO_2 incubator at 37°C. The culture medium was changed every other day. Morphologically, the MCs formed a confluent monolayer of cells with a typical cobblestone appearance in about 5 days. The identity of the MCs was controlled with the demonstration of the presence of intracellular cytokeratins 6 and 18 and of the absence of von Willebrand factor with monoclonal antibodies (Dako-Ck1, LP 34, Dako, Glostrup, Denmark).[13,14]

Microvascular endothelial cells. Human subcutaneous fat was obtained through liposuction or abdominoplasty. The protocol that was described by Williams et al[16] was followed with some minor modifications. In the case of abdominoplasty, the fat tissue was minced with a scalpel or scissors to a size that was comparable with the liposuction material. After 1 wash step with sterile PBS, the fat was transferred to a prewarmed (37°C), filtered collagenase solution that contained 4 mg/mL of collagenase type I (Sigma, St Louis, Mo) and 4 mg/mL of bovine serum albumin (Sigma) in a PBS (10 mL of fat + 10 mL of collagenase per tube). This was followed by a 30-minute incubation with gentle shaking at 37°C. The resultant slurry was sifted through a 1-mm sieve to eliminate the resting lumps and the fibrous strands, after which it was centrifuged at 1200 rpm for 10 minutes. The adipocytes at the top of the tube and the collagenase solution were decanted, and the pellet was resuspended in M199, which contained penicillin (100 units/mL), streptomycin (100 g/mL), and amphotericin B (4 μg/mL, M199; Gibco). The cell suspension was centrifuged once more at 1000 rpm for 5 minutes, after which it was resuspended in 12 mL of tissue culture medium (M199, with aforementioned antibiotics, 20% FCS, 5 IU/mL heparin [Leo Pharmaceutical, Weesp, The Netherlands], and 50 μg EC growth factor [prepared in-house from bovine hypothalamus]). The cells were plated onto the 75-cm^2 fibronectin precoated polystyrene flasks and grown in a fully humidified 5%-CO_2 incubator at 37°C. The culture medium was changed every other day. The morphology varied between cobblestone and more elongated forms, and the identity of the cells was controlled with the demonstration of positive immunostaining for endothelial markers—von Willebrand factor, Ulex Europeaus 1, CD-31, EN-4.

Human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVECs)

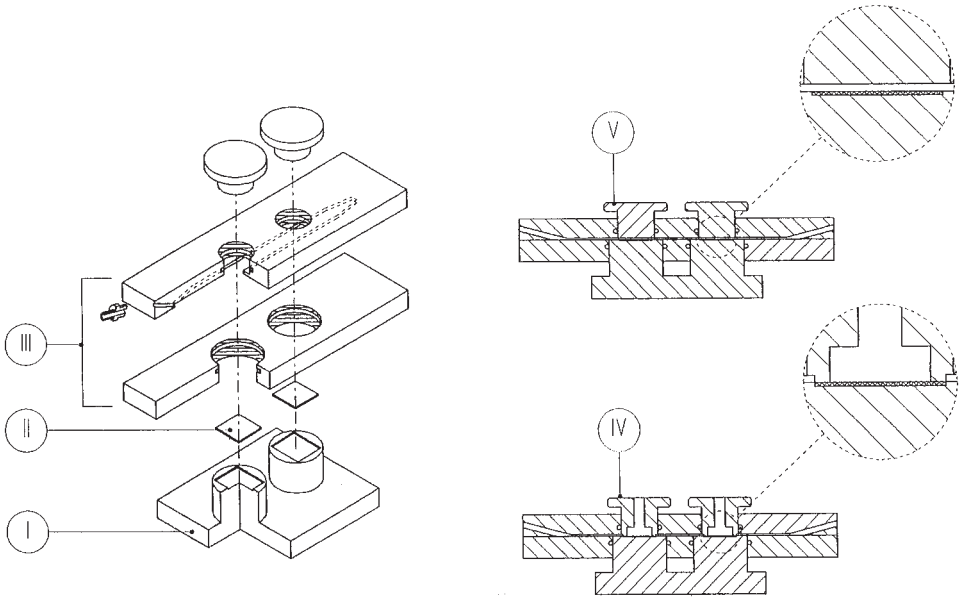

Fig 1. Diagram of seeding/perfusion chamber. **I,** Large caps on which pieces of ePTFE prostheses **(II)** were immobilized. The rectangular chamber **(III)** was placed over these 2 caps, which exposed 10 mm × 18 mm of graft surface to flow. The seeding caps **(IV)** or perfusion knobs **(V)** could be placed in upper side of chamber.

were isolated according to Jaffe et al[23] with some minor modifications.[24,25] The HUVECs were identified by the presence of von Willebrand factor. The HUVECs were grown in RPMI-1640 (Gibco), with 20% human serum (HS; pooled from 20 healthy donors) and antibiotics with the same conditions as were described for the culture of the MCs. They formed a monolayer in about 7 to 10 days.

Human adult endothelial cells. The HAECs were isolated from pieces of human aorta. The pieces of aorta were placed in prewarmed trypsin for 15 minutes, with the luminal surface facing downwards. The luminal surface then was scraped gently with a rubber policeman and rinsed with the culture medium. The mixture of trypsin, culture medium, and cells was centrifuged at 1100 rpm for 5 minutes. The cell pellet was resuspended in RPMI-1640, which contained 10% HS, 0.15 mg/mL EC growth supplement (obtained from bovine hypothalamus), 5 IU/mL of heparin, and antibiotics (penicillin, streptomycin, and amphotericin B). The cells were cultured in a 25-cm^2 culture flask (Costar) with the same conditions as were described above. A monolayer was formed in about 2 weeks. The second passage cells were used for the experiments and were identified by the presence of von Willebrand factor. At least 24 hours before a perfusion experiment, the culture medium for HUVECs and HAECs was changed to M199 with 10% FCS, which was the same as was used for the MCs. In some experiments, the cells were incubated with a culture medium that contained 4 mmol/L of allopurinol (Sigma) or with a culture medium that contained 10% HS, for a time period of 24 to 48 hours before a perfusion.

Perfusion chamber

For the perfusion experiments, we used a newly developed chamber (Fig 1) on the basis of a well-described and often-used model.[26,27] Two pieces of sterile expanded polytetrafluoroethylene (ePTFE) vascular grafts (Fig 1, II; W. L. Gore & Assoc, Flagstaff, Ariz) of standard thickness (0.64 mm) and internodal distance (30 μm) were cut (18 mm × 21 mm) and immobilized on the 2 large caps (Fig 1, I) with the luminal surface facing upwards. The rectangular chamber (Fig 1, III) was placed over these 2 caps and sealed with rubber O-rings. On the upper side of the flow chamber, 2 smaller holes were made in which either the seeding cap (Fig 1, IV) or the perfusion cap (Fig 1, V) could be sealed. In the seeding caps, a small rectangular chamber (1.25 cm^2) that could be placed on the ePTFE graft surface was made, which allowed the cells to be seeded on a precisely described area completely in the lane of the flow. After a seeding proce-

dure, the seeding caps were changed for the perfusion caps, which gave the roof of the perfusion chamber a smooth surface. The surface of each of the pieces of ePTFE graft exposed to the flow measured 10 mm × 18 mm, and the height of the flow chamber measured 1.0 mm.

Graft seeding

Immediately before an experiment, a monolayer of MCs, MVECs, HUVECs, or HAECs (all second passage) was detached by trypsinization, counted with a Coulter Counter (Coulter Electronics Ltd, Luton, United Kingdom), and resuspended in M199 that contained 10% FCS (with or without allopurinol, as stated) or 20% FCS, depending on the tissue culture medium. The cylindrical hole (diameter, 6 mm) in the seeding cap gave access to the rectangular chamber, with 1.25 cm^2 of the prosthetic material exposed. Fibronectin was purified from human plasma as described before[28] and was used to precoat the grafts (50 μg/mL = 20 $\mu g/cm^2$) for 1 hour at 37°C. The MCs, MVECs, HUVECs, and HAECs were seeded (2×10^5 cells/cm^2) and allowed to attach for 4 hours. Subsequently, the seeded grafts were rinsed thoroughly with N-2-hydroxyethylpiperazine-N-2-ethanesulfonic buffered saline solution (HBS; 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic, 145 mmol/L NaCl, pH 7.4) to remove the unattached cells, the seeding cap was exchanged for the perfusion cap, and the perfusion chamber was connected to the flow circuit. The number of cells that were attached to the prostheses at the beginning of the perfusion experiments and the detachment of seeded cells as a result of shear forces have been studied before and were described in detail elsewhere.[21,22]

Flow circuit and perfusions

With the change of the seeding cap for the smooth perfusion cap, the seeded grafts could be perfused without manipulation, with possible cell loss as a consequence. The hemodynamic parameters of the flow circuit were calculated with the work of Milnor[29] and Muggli et al.[30] The maximum Reynolds number in our flow circuit was 95, so the calculations could be on the basis of laminar flow. Two different perfusion models were studied: a model in which recirculated, marginally anticoagulated blood was used; and an ex vivo model in which non-anticoagulated blood was used.

Recirculation perfusions. The flow circuit as used for the recirculation perfusions is shown in Fig 2. A 50-mL tube of freshly drawn blood from healthy donors who denied the use of any medication for at least 2 weeks before the donation. The blood was anticoagulated with low molecular weight heparin (20 U/mL; Fragmin, Kabi Pharmacia AB, Sweden) and was placed in a waterbath at 37°C. The cap of the 50-mL tube had 1 inlet port and 1 outlet port. The outlet port was connected to a roller pump, which was capable of delivering flow rates between 2.8 and 280 mL/min (model 2010, Verder, Vleuten, The Netherlands). To achieve a steady flow, a funnel was connected between the roller pump and the perfusion chamber. The distal end of the perfusion chamber was connected to the inlet port to complete the circuit. All of the connections were made with a silicon tubing. The experiments were done at a shear rate of 400/s (38 mL/min), which is characteristic for medium-sized arteries. Before each experiment, the pump speed was calibrated and a new tubing was used. Before use, the new tubing first was rinsed with HBS. Recirculation perfusions were performed for 10 minutes. After perfusion, the whole system was rinsed with 50 mL of prewarmed HBS. The pieces of seeded ePTFE graft were collected and fixed for scanning electron microscopic (SEM) evaluation.

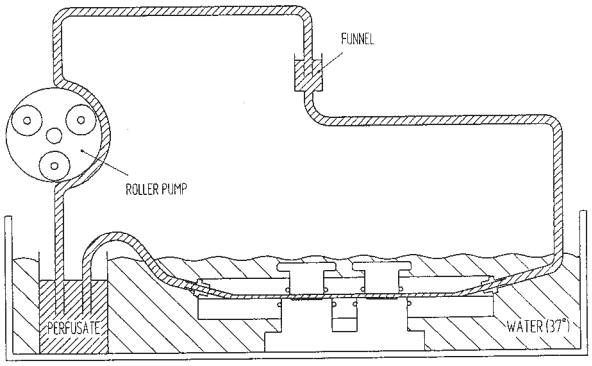

Fig 2. Diagram of flow circuit that was used for recirculation perfusions.

Ex-vivo perfusions. Non-anticoagulated blood was drawn directly through the chamber from the antecubital veins of healthy donors with a 19-G needle[31] (Fig 3). The blood was aspirated over the seeded prostheses at a constant flow rate of 10 mL/min with a syringe pump (Harvard Apparatus, South Natick, Mass) that was placed distally to the perfusion chamber. The blood flow rate of 10 mL/min resulted in a wall shear rate of 100/s at the cell layer on the prostheses. This shear rate reflects the flow conditions in the veins and favors fibrin-rich clot formation.[32] All of the healthy volunteers denied taking any medication in the 14 days before the donation. The platelet

Fig 3. Ex vivo perfusion. Non-anticoagulated blood is drawn from antecubital vein through perfusion chamber. Blood samples are taken through tubing. After the experiment, perfused graft will be taken out of perfusion chamber and fixed for scanning.

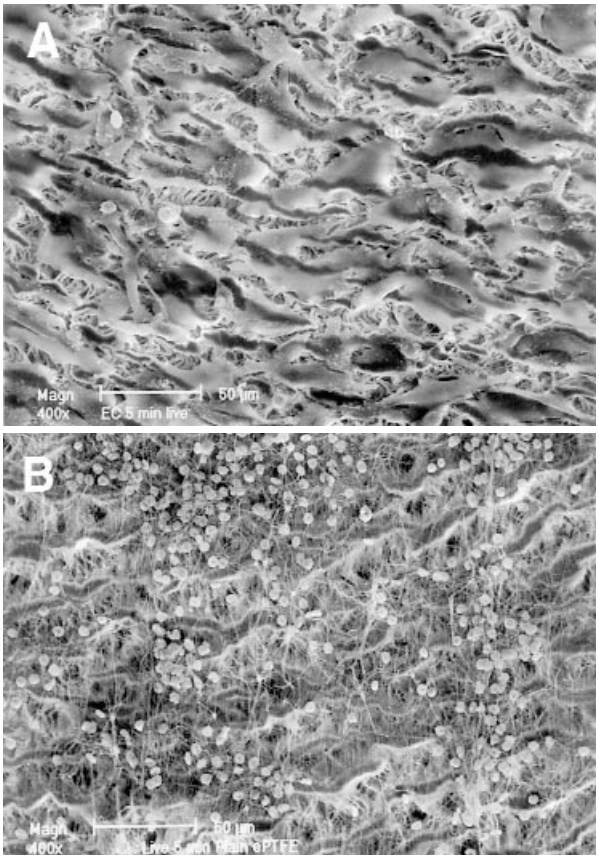

Fig 4. Surfaces of ePTFE graft perfused for 5 minutes with non-anticoagulated blood (shear rate 100/s). **A,** ePTFE graft seeded with MVECs demonstrates almost no fibrin generation and minimal thrombocyte aggregation. **B,** Standard plain ePTFE graft shows fibrin formation and thrombocyte aggregation.

counts, the leucocyte counts, and the hematocrit were all within the normal ranges. At the end of a 5-minute perfusion, the prewarmed HBS was perfused at the same flow rate for 40 seconds. The flow chamber was disconnected, and the pieces of ePTFE graft vascular prostheses were fixed for evaluation with SEM.

Determination of thrombin and fibrin formation

For the in vitro perfusions, the blood samples (450 µL) were collected from the container immediately before the recirculating perfusions (0-value), after every minute up to 5 minutes, and after 10 minutes during the experiments.

During the ex vivo perfusions, 450-µL blood samples were collected directly through the wall of the silicon tubing with a 25-G needle. The first sample was taken proximal to the perfusion chamber after the first few mililiters had passed (0-value), and samples then were taken directly distal to the perfusion chamber after every minute. At the end of the perfusion, another proximal sample was collected. All of the samples were mixed immediately with 50-µL of ethylenediamine tetraacetic acid (100 mmol/L) and centrifuged at 3500 rpm for 5 minutes, and the aliquots of plasma were stored at –20°C until assayed.

Prothrombin fragment 1+2 (F 1+2) was measured with a commercially available radioimmunoassay (Enzygnost–F 1+2, Behring, Germany). A radioimmune assay kit was used for the fibrinopeptide A (FPA) measurements (RIA-mat FPA, Byk-Sangtec GmbH & Co KG, Dietzenbach, Germany).

As a control, both the recirculating and the ex vivo perfusions were performed with blank ePTFE graft prostheses (without cells). To determine the amount of F 1+2 that was generated during a perfusion experiment, the 0-value was subtracted from the amount of F 1+2 that was measured in all of the other samples from the same experiment.

Morphologic examination

Each graft that was prepared for SEM was rinsed with HBS, fixed in 2% glutaraldehyde for 1 hour, postfixed in 1% osmium tetroxide (Sigma) for 1 hour, and then dehydrated through increasing concentrations of ethanol (50% to 100%). The samples were critical-point dried (CPD 010, Balzers Union, Lichtenstein),

Table I. Recirculation perfusion: plasma levels of prothrombin fragment 1+2, and fibrinopeptide A after 5 and 10 minutes (mean ± standard deviation of the mean)

			F 1+2 (nmol/L)		*FPA (ng/mL)*	
Cell types	*Additions*	*N*	*5 minutes*	*10 minutes*	*5 minutes*	*10 minutes*
MC	—	5	2.40 ± 0.58	4.38 ± 1.1*	197 ± 101	362 ± 51
	Allopurinol	5	0.43 ± 0.29	0.70 ± 0.38	NI	NI
HAEC	—	4	0.66 ± 0.21	1.86 ± 0.22	NI	NI
	Allopurinol	4	0.78 ± 0.24	1.85 ± 0.43	NI	NI
MVEC	—	10	0.47 ± 0.17	0.70 ± 0.30†	NI	NI
HUVEC	—	5	0.22 ± 0.16	0.26 ± 0.07†	NI	NI
No cells	—	4	0.19 ± 0.09	0.25 ± 0.07†	NI	NI

F 1+2, prothrombin fragment 1+2; *FPA,* fibrinopeptide; *N,* number of perfusions; *MC,* human mesothelial cells; *HAEC,* human adult endothelial cells; *MVEC,* human microvascular endothelial cells; *HUVEC,* human umbilical vein endothelial cells; *NI,* no increase measured.
* $P = .01$, compared with mesothelial cells with allopurinol.
† $P < .05$.
The total amount of prothrombin fragment 1+2 generated during the 10-minute perfusion was calculated by multiplying the end-concentration of prothrombin fragment 1+2 by the total volume (50 mL). Recirculation perfusions were performed with marginally anticoagulated blood with a flow rate of 38 mL/min (shear rate, 400/s).

sputtered with gold (SCD 040, Balzers Union), and finally examined with SEM (Camscan, Cambridge, United Kingdom).

Statistical analysis

The results are expressed as the mean ± the standard deviation of the mean, unless otherwise stated. A nonparametric test was used to determine the significant differences between the groups (Mann-Whitney test). A P value of $< .05$ was considered significant.

RESULTS

Recirculation perfusions

Procoagulant activity of different surfaces. A summary of the amount of F 1+2 that was generated after 5 and 10 minutes of perfusion with the most relevant conditions is shown in Table I. The MCs generated the highest values of F 1+2 in 10 minutes (4.38 ± 1.1 nmol/L). When allopurinol was added to the culture medium to reduce the tissue factor expression, significantly less F 1+2 was generated by the MCs (0.70 ± 0.38 nmol/L; $P = .01$, vs FCS). When the MCs were cultured in the tissue culture medium that contained 10% HS instead of 10% FCS, the F 1+2 values rose significantly (6.25 ± 0.52 nmol/L).

The HAECs that were seeded on the ePTFE graft prosthesis generated significantly lower amounts of F 1+2 than the MCs that were cultured in FCS (1.86 ± 0.22 nmol/L; $P < .05$) but significantly higher amounts when compared with the MCs that were treated with allopurinol ($P < .05$). The HAECs did not respond to the allopurinol treatment. The MVECs that were seeded on the ePTFE graft generated comparable amounts of F 1+2 (0.70 ± 0.30 nmol/L) to the MCs that were treated with allopurinol (0.70 ± 0.38 nmol/L).

The lowest levels of F 1+2 were measured after the perfusion of the prostheses that were seeded with HUVECs (0.26 ± 0.07 nmol/L) or when the blank prostheses were perfused (0.25 ± 0.07 nmol/L). These F 1+2 values were significantly lower than all of the others ($P < .05$ vs MCs with allopurinol or MVECs).

High amounts of FPA were generated after 5 and 10 minutes by the MCs that were cultured with 10% HS or 10% FCS (836 ± 76 ng/mL and 362 ± 51 ng/mL, respectively, after 10 minutes). No increase in the FPA levels was seen during all of the other experiments. The mean FPA value in the blood before perfusion was 14 ng/mL (range, 9 to 22 ng/mL), which is the same as was described by others.[33]

Morphologic examination. All of the flow surfaces were fixed and examined with SEM. After the perfusion of the MCs, which were cultured in the presence of 10% FCS or 10% HS, a layer of fibrin was deposited parallel to the blood flow on the complete surface of the seeded prostheses, with platelet aggregates adhering to the fibrin strands and red blood cells captured in the fibrin meshwork. When allopurinol was added to the culture medium, significantly less fibrin or aggregates were found. In the cases of ePTFE grafts that were seeded with MVECs, HUVECs, or HAECs, there were clearly less fibrin and thrombocyte aggregates when com-

Table II. Ex-vivo perfusion: plasma levels of prothrombin fragment 1+2 and fibrinopeptide A after 1 and 5 minutes (mean ± standard error of the mean)

		F 1+2 (nmol/L)			*FPA (ng/mL)*	
Cells	*N*	*1 minute*	*5 minutes*	*AUC (nmol/L·minutes)*	*1 minute*	*5 minutes*
MC	4	3.37 ± 2.47	3.0 ± 1.61	14.44 ± 2.9	1953 ± 154	1189 ± 308
MC+All	5	1.48 ± 1.8	2.43 ± 2.1	10.67 ± 3.6	1030 ± 535	1835 ± 419
MVEC	10	0.06 ± 0.02	2.70 ± 0.79	7.10 ± 2.2	38.4 ± 16.4	527 ± 109*
HAEC	5	0.05 ± 0.03	4.47 ± 1.57	8.46 ± 2.7	22 ± 10.7	758 ± 173*
HUVEC	5	0.19 ± 0.08	1.70 ± 0.49	1.49 ± 0.42	222 ± 88	696 ± 344*
Plain ePTFE	4	0.09 ± 0.14	0.25 ± 0.07	0.93 ± 0.33	16 ± 4	1653 ± 550

F 1+2, prothrombin fragment 1+2; *FPA,* fibrinopeptide; *N,* number of perfusions; *MC,* human mesothelial cells; *HAEC,* human adult endothelial cells; *MVEC,* human microvascular endothelial cells; *HUVEC,* human umbilical vein endothelial cells; *NI,* no increase measured.

* $P = .01$, compared with mesothelial cells with allopurinol.

† $P < .05$.

The total amount of prothrombin fragment 1+2 generated during the 10-minute perfusion was calculated by multiplying the end-concentration of prothrombin fragment 1+2 by the total volume (50 mL). Recirculation perfusions were performed with marginally anticoagulated blood with a flow rate of 38 mL/min (shear rate, 100/s).

pared with non-allopurinol treated MCs. The unseeded ePTFE grafts showed higher platelet aggregation than the seeded grafts.

Ex vivo perfusions

Thrombin generation. To better evaluate the non-thrombogenic properties, the seeded grafts were subjected to ex vivo perfusions, with non-anticoagulated blood. Pieces of the ePTFE grafts were seeded with MCs (with or without allopurinol), with MVECs, with HAECs, or with HUVECs. The blank pieces of the ePTFE graft prostheses were taken as controls.

During the ex vivo perfusions, 7 samples were taken by puncture through the wall of the silicon tubing—2 proximal to the chamber and 5 distal to the chamber, all close to each other.

Table II shows the generation of F 1+2 and FPA during the ex vivo perfusions. Although a large variation was seen within each of the 6 experimental conditions, the MCs generated the highest levels of F 1+2.

For the MCs, the F 1+2 values were maximal within 1 or 2 minutes without an increase or reduction during the following minutes. The inhibitory effect of allopurinol on F 1+2 generation was less clear in these experiments and, at certain time points, non-existing. When MVECs, HAECs, HUVECs, or plain prostheses were used, lower values of F 1+2 were found, although all of the conditions clearly showed generation of F 1+2 towards the end of the experiments.

The concentration of F 1+2 that was measured proximal to the chamber (t = 0) was always below 1.1 nmol/L and was subtracted from all other values from the same donor. The F 1+2 concentration that was measured in the control samples taken proximal to the chamber at the end of the perfusions (t = 5 minutes) was never more than 0.2 nmol/L above the value at t = 0. So the generation of F 1+2 during an experiment was mainly a result of the (seeded) prostheses in the chamber.

In the case of the perfusion of MCs or of MCs and allopurinol, the FPA levels corresponded with the rise in F 1+2. The HUVECs, the HAECs, and the MVECs, however, did not translate their higher levels of F 1+2 at 5 minutes into such a high rise in FPA as seen with the MCs or with the plain ePTFE grafts ($P < .05$ MCs, MCs and allopurinol, or ePTFE grafts vs HAECs, HUVECs, or MVECs).

Morphologic examination. After the perfusion of the seeded MCs, a thick layer of fibrin covered the complete surface of the prostheses, with platelet aggregates adhering to the fibrin meshwork. No morphologic differences were seen when the MCs were treated with allopurinol before the perfusion. After the perfusion of the EC-seeded prostheses (HUVECs, HAECs, and MVECs) or of the plain ePTFE graft prostheses, the fibrin strands, although in lesser numbers as compared with the MCs, were seen all over the perfused surface in the direction of the flow, but the cells or the plain prostheses were clearly visible.

DISCUSSION

The original idea of EC seeding was the improvement of the patency of prosthetic small-diameter grafts by the establishment of a biologic

antithrombotic lining on the luminal surface of the graft. This process has reached the phase of clinical evaluation, and encouraging results have been reported.[34,35] The major problems in all of these studies were the limited availability and the poor growth of the human HLA-compatible ECs. The MCs came into focus as a possible alternative because they are anti-thrombotic, fibrinolytic, and anticoagulant in vivo.[7-13] Unfortunately, procoagulant activity is induced on MCs by isolation and culture and was found to be caused by an increased expression of tissue factor.[13] We have shown that tissue factor expression can be decreased to the same level as measured on adult ECs by changing culture conditions and inhibiting the formation of free radicals with allopurinol, a xanthine oxidase inhibitor.[14] The microvascular cells that were isolated from fat tissue are another alternative to adult ECs. They are abundantly available in human beings, and they represent a source of cells large enough for single-stage cell seeding without the need for prior culture. In this study, we investigated the net result of procoagulant and anticoagulant activities of different available cell types that were seeded on vascular prostheses under flow conditions with whole blood.

For the perfusion experiments, we first used low molecular weight heparin–anticoagulated whole blood and measured the prothrombin activation (measured by F 1+2) and thrombin cleavage of fibrinogen (measured by FPA). We demonstrated that the MCs that were cultured in medium that contained 10% FCS or HS are highly procoagulant because high levels of F 1+2 and FPA were found and 100% fibrin-coverage of the cells was seen by SEM. The generation of F 1+2 was linear in time, and a mean F 1+2 generation of 4.38 nmol/L after 10 minutes meant that about 0.3% of the total amount of prothrombin (physiologic concentration, 1.4 μmol/L) was activated after 10 minutes. When allopurinol was added to the culture medium and the cells were allowed to attach for 4 hours, 0.7 nmol/L of F 1+2 was generated, and no fibrin was seen with SEM. Apparently, 0.05% activation of prothrombin was insufficient for fibrin generation.

Although the tissue factor expression of the allopurinol-treated MCs equalled the level that was measured on the cultured adult ECs,[14] the generation of F 1+2 was significantly lower. A possible mechanism for this could be the high expression of thrombomodulin on MCs, which we demonstrated before.[36]

In the ex vivo perfusion system, we could measure the net result of the procoagulant and anticoagulant properties of seeded cells to its full extent because no anticoagulants were present. We chose to study the following cell types that showed the lowest F 1+2 generation after seeding and perfusion with anticoagulated blood: HUVECs, which are known to express virtually no tissue factor when unstimulated;[37] MVECs, which we demonstrated to be comparable with HUVECs concerning tissue factor expression; and MCs that were treated with allopurinol. Furthermore, MCs that were grown in 10% FCS were chosen as a positive control because we demonstrated previously that these cells express substantial amounts of tissue factor.[13] The plain ePTFE graft was chosen as control.

The significant decrease in procoagulant activity when MCs were treated with allopurinol could not be seen during the ex vivo perfusions.

The plain ePTFE graft prostheses performed well during the first few minutes of the ex vivo perfusion. Yet after a few minutes, the activation of prothrombin started, high levels of FPA were found, and SEM showed fibrin strands and platelet adherence on the surface of the prostheses after 5 minutes. This underlined the need for an antithrombotic biologic lining on the graft. When the HUVECs, HAECs, and MVECs were seeded and perfused, generation of F 1+2 and FPA also was found. Although this generation started earlier than when the plain ePTFE graft was perfused, it did not show the enormous increase in both F 1+2 and FPA towards the end of the experiment. Significant differences between the 2 surfaces therefore may only be obtained when longer ex vivo perfusions are performed.

The plain ePTFE graft performed well with respect to its procoagulant activities when perfused with anticoagulated blood, but, when native blood was used, it performed poorly. This observation would lead one to conclude that the study of graft performance with anything but non-anticoagulated blood might lead to misleading results.

We believe that there is no intrinsic difference in surface procoagulant effect between the 2 perfusion models that we used. However, it is likely that the observations in the recirculating perfusions are mainly heparin-induced. In the ex vivo model, there is no inhibition of coagulation, which makes it a more sensitive model to look at any existent procoagulant effect.

With a perfusion system that was developed to test vascular grafts in a human system, we demonstrated in this study that ePTFE vascular grafts that are seeded with MCs are highly procoagulant. We also showed that plain prostheses initiate coagulation during perfusions with native blood. We therefore think that the

patency rates of small-diameter prostheses can indeed be improved by lining them with a biologic lining. In this study, we only measured early events and not a steady state. To perform longer perfusions, which are necessary to study steady state levels, one has to anticoagulate. This will, however, have a drastic effect on the predictability. Despite the theoretical advances of MCs over ECs, they do not seem to perform satisfactorily in vascular surgery, unless their procoagulant properties are seriously downregulated. HUVECs perform well in vitro, have a relatively quick growth rate but obviously cannot be harvested in the group of patients in need of vascular surgery. Adult ECs still are used most often and are considered the gold standard by most cell-seeding groups. Microvascular ECs, however, are available in much larger numbers through minimally invasive surgery, can be cultured first or used immediately, and show comparable results with adult ECs when seeded.

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